COMPLETE FRACTIONATION OF BACTERIOCHLOROPHYLL AND ITS DEGRADATION PRODUCTS

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SUMMARY

1. With small amounts (approx. 100 mg) of Rhodospirillum rubrum two rapid methods for complete separation of the bacteriochlorophyll and its degradation products are described.

2. Absorption coefficients of the bacteriochlorophyll fractions were calculated from an accurate and sensitive fluorometric analysis of magnesium and the physical values were compared with those previously published.

3. Fluorescence, phase test, and HCl numbers studied with each component are presented.

4. After chemical and photooxidation, several bands of green pigments and other degradation products were produced. The physical data obtained from the four green pigments were quite similar. The possible sequence of production of these oxidized products is described. The mechanism of oxidation and the chemical structures of the individual products are not known.

Running Head: Complete Fractionation of bacteriochlorophyll

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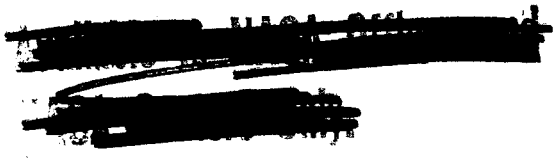
5. The fast migrating gray-green fraction showed a distinguishing gamma band at 280 mμ with less prominent Soret and red bands.

INTRODUCTION

After the classic bacteriochlorophyll studies by van Niel and Arnold[1] and Fischer and Stern[2], Holt and Jacobs[3] and Smith and Benitez[4], using the sucrose column method, purified this unstable pigment. The technique has been applied as standard procedure for the purification of bacteriochlorophylls (Goedheer[5], Strain et al[6]) and chlorophylls (Anderson and Calvin[7]).

The sucrose column method is, however, quite laborious and time consuming and the repeated chromatography involved in the process of purification inevitably gives rise to large proportions of degradation products which are difficult to separate from the bacteriochlorophyll. To improve these techniques and achieve a complete separation of the bacteriochlorophyll and its degradation products, a number of previously published methods of chlorophyll fractionation were tried,



including the sucrose plate method of Colman and Vishniac[8] and the paper chromatography method of Jensen, Aasmundrud, and Eimhjellen[9]. These methods were found to be largely unsatisfactory for the complete separation of the various products arising from bacteriochlorophyll.

This paper presents two rapid and simple methods which will effect a complete separation of bacteriochlorophyll "a" and its degradation products, along with some data obtained from studies of absorption, fluorescence, and other physical-chemical properties of these components using less than 0.1 g of lyophilized or 1.0 g of wet bacterial samples. An abstract on this subject has been presented by Kim and Feller[10].

MATERIALS AND METHODS

Rhodospirillum rubrum (ATCC No. 277) was cultured in cotton-plugged 500-ml erlenmeyer flasks containing 300 ml of trypticase soy broth under approximately 200 foot-candles of incandescent light in a 26° C incubator. For extraction of bacteriochlorophyll, 5 to 8-day cultures in logarithmic growth phase were used. For routine isolation and degradation studies, about 1 ml

of 3 X washed, packed cells or 100 mg batches of lyophilized samples were extracted with 10 ml of methanol. Reagent grade solvents, such as methanol, acetone, and petroleum ether, were redistilled prior to usage. The ether was treated to be peroxide free prior to usage. For the analysis and preparation of bacteriochlorophyll components, two chromatographic techniques were employed, either column or thin layer. Columns (1 x 20 cm) were made of dry-packed 120/140 mesh Gas Chrom P (Applied Science Laboratories, Inc., Pa.). For thin-layer chromatography, 7.5 in. square plates of powdered Gas Chrom P or Kieselguhr G (Brinkman Instruments, Inc., N. Y.) impregnated with 7% triolein in petroleum ether (30-60° b.p.), were used. For absorbance analysis, the Hitachi 139, Perkin-Elmer 350, and Cary 14 spectrophotometers were used. Fluorescence measurements were made with the Turner 210 and the Baird Atomic SF 1 fluorospectrophotometers. Magnesium contents of pigments were analyzed by a modified fluorometric method of Schachter[11] using the Turner Fluorometer Model III. Lipid and fatty acid contents of the bacteriochlorophyll were analyzed by gas chromatography using an ethylene glycol adipate column and measuring effluents with a calibrated ionization detector.

EXPERIMENTAL AND RESULTS

Column chromatography

Figure 1 shows a flow diagram of bacteriochlorophyll fractionation. Twenty milliliters of methanol containing 1 ml of 1 M ascorbate was added to 1 ml of packed cells or 100 mg of dried cells in a centrifuge tube of 50-ml capacity. The cells were well suspended and centrifuged immediately at 30 000 g × minutes. The blue-green supernatant was shaken vigorously for 2 to 3 min. in a 500-ml separatory funnel with 50 ml of petroleum ether plus 3 ml of distilled water. The upper petroleum ether phases from four additional extractions were pooled. Five extractions were necessary to decolorize the aqueous layer. The extracts were evaporated to a small volume (about 2.0 ml) under reduced pressure. A 25 × 1-cm glass tube, plugged with glass wool at the tapered 3.0-mm-diameter end, was dry packed with small amounts (< 2 ml) of Gas Chrom P to about 20-cm height, tamping with a glass rod each time to ensure uniformity of the column.

The column was placed in a dark, ventilated hood and the collector was connected to a vacuum line to exert a gentle suction throughout the column. After the small sample placed on top of the column moved into the bed, acetone-petroleum

ether solutions (see Fig. 1) were successively added to elute the bacteriochlorophyll components. Since all the discrete fractions were clearly visible, having different rates of migration, elutions could be conveniently regulated to optimize rapid but effective resolution by pinch cock control of the vacuum line. The fractionation was generally completed within 1 hr, but unresolved eluates should be concentrated and rechromatographed on smaller columns. Carotenoid fractions were first eluted and discarded. Of three successive fractions separated, the pinkish-violet fraction (F-1) was pheophytin, the blue fraction (F-2) was bacteriochlorophyll, and the gray-blue fraction (F-3) appeared to be an oxidized component of F-2 (Fig. 4). Each fraction was vacuum-dried and dissolved in ether or other solvents for further analysis.

Thin-layer chromatography

For analytical and preparational runs of thin-layer chromatography, thin layers of 0.25 mm thick and 0.50 to 0.75 mm thick were used, respectively. After examination of various thin-layer materials in many solvent systems, two different thin-layer chromatographic methods were

found to be most effective both for analysis and preparation of the bacteriochlorophyll and its degradation products. The powdered Gas Chrom P plates with the solvent, acetone-petroleum ether in a 5/95 volume ratio, separated the bacterial pigments extracted in methanol in the following order of proximity to the spot origin: F-3, F-2, green fractions (when oxidized), F-1, and carotenoids. The Kieselguhr G plate coated with 7% triolein in petroleum ether, on the other hand, completely reversed the order of separation obtained by former methods in the solvent of methanol-acetone-water in a 20/4/3 volume ratio. The coating of triolein is essential for the separation, but to be more effective, spotting the sample should be done on the uncoated area of the 1.5-in. margin of the thin-layer plate.

The above two-thin-layer chromatographic methods could alternatively be used for further purification and confirmation of the identity of the fractions. Several such fractions isolated and described below were run through these two chromatographic systems. Occasionally, polyvinyl and sugar plates, using solvents of 70 to 80% acetone in water for the former and 1 to 5% acetone in petroleum ether for the latter, were also applied for certain fractions.

The Kieselguhr plate was better than the Gas Chrom P plate in that it gave more discrete separation of fractions, especially the greenish oxidized components, G-1, G-2, G-3, and G-4.

Figure 2 shows a summary of complete fractionation of bacteriochlorophyll and its degraded components on thin-layer plates of Kieselguhr G impregnated with triolein. The chromatograph was run in a dark chamber at room temperature for 45 to 60 minutes. The fresh methanol extract shows 3 pinkish, narrow bands near the origin and a large blue band in the plate center. The two slowest moving pink bands on the margin of triolein are carotenoids, the third pinkish violet band is pheophytin (F-1), and the blue band is bacteriochlorophyll (F-2).

When the methanol extract was exposed to the dim light for some time or aged in a dark refrigerator for several days, a light green fraction (G-2) right behind F-2, a gray-blue fraction (F-3) ahead of F-2, and a fast migrating gray-green fraction (GG) appeared in addition to the components found from the fresh methanol extract. If the same methanol extract was vigorously aerated in the dark for 30 min. or addition of periodate, another green fraction (G-1) appeared right behind G-2.

Exposure to bright incandescent light (100 W × 2 min. at 10-cm distance) in the air or addition of potassium permanganate or iodine (4 umole per umole of bacteriochlorophyll) gave nearly similar patterns of degradation as the aerated sample which, in addition, contained F-3 component. Longer light treatment of the methanol extract in air (100 W × 10 min. or longer) caused a complete degradation of F-2 and the production of two additional green bands (G-3, G-4) which travel faster than G-2. When ascorbic acid (100 umole per umole of bacteriochlorophyll) was added to the extract, no green bands, nor F-3 band, appeared even after the light treatment (100 W × 2 min.), but the GG fraction did appear at the solvent front.

Absorption spectra

Preparative thin-layer chromatography was run on 0.5 mm thick Kieselguhr plates and the separated bands were scraped off, thoroughly vacuum-dried over calcium chloride, and eluted with ether. Figure 3 shows the absorption spectra of F-1 (pheophytin), G-2 (the heaviest green fraction), F-2 (bacteriochlorophyll), and GG (gray-green fraction).

The absorption bands of F-1 in ether were characteristic to the bacteriochlorophyll "a" pheophytin showing three main peaks at 750, 528, and 357 mμ and some minor ones at 680,

616, 494, 461, and 387 mμ. The ratio of absorbances between the Soret (357 mμ) and red (750 mμ) bands was calculated to be 1.65. The F-2 fraction in ether also showed three main absorption bands at 770, 575, and 358, but the ratio of absorbances between Soret (358) and red (770 mμ) bands was only 0.76. Besides, this bacteriochlorophyll fraction showed a shoulder at 679 mμ and a Soret branch at 391 mμ. All the green fractions (G-1, G-2, G-3, G-4) separated by thin-layer chromatography gave an identical absorption spectrum in ether showing two distinguishing peaks at 675 and 435 mμ with three minor bands at 625, 585, and 537 mμ and a shoulder at 385 mμ. The absorption spectrum of GG fraction in ether was quite characteristic in that it had a huge gamma band at 280 mμ and relatively smaller bands scattering over the entire range of scanned spectrum showing blunt peaks at 330, 415, 634, 675, and 770 mμ.

Figure 4 shows the absorption spectrum of the gray-blue fraction (F-3) which is compared with that of bacteriochlorophyll (F-2). General absorption patterns of both spectra are quite similar, but they are different in that the absorbance ratio 358/770 in F-3 becomes close to unity, and shoulders at 675 and 435 mμ of F-3 become

more prominent. This F-3 fraction appears on the Kieselguhr thin-layer plate as a grayish blue spot migrating some distance ahead of F-2. Its color changed readily into light green even under the weak light. Owing to this extreme lability, the absorption coefficients of F-3 were not calculated. The results of the phase test were positive and the HCl number was 30. Thus, F-3 may be an immediate precursor of the further oxidized product, G-2.

Magnesium analysis

The gravimetric method used for the calculation of the absorption coefficients of bacteriochlorophyll (Holt and Jacobs[3]) was not applicable for the small sample applied for the present study. The colorimetric method of magnesium analysis described by Smith and Benitez[4] was tried initially, but abandoned later because the method was found to be not only laborious but also insensitive, requiring as much as 5 to 120 μg of magnesium.

The magnesium contents of bacteriochlorophyll were conveniently analyzed by the fluorometric technique of Schachter[11] who originally described it to analyze the magnesium contents in blood serum and urine. With minor modification of the original method, a highly reproducible and linear standard curve was obtained with the magnesium contents ranging from 0.0 to 1.0 μg in the final 2.4-ml volume by use of the Turner Model III, Fluorometer.

Samples of purified bacteriochlorophyll of known absorbances were diluted in ethanol so that the magnesium contents would reach around 0.5 to 1.0 μg range. Tentative calculations of the magnesium and bacteriochlorophyll contents were made on the basis of the absorbance of peak 770 mμ and its specific absorption coefficient (100 $cm^2 \times g^{-1}$) in ether published earlier by Smith and Benitez[4].

The 430 to 435 mμ excitation may also have caused the bacteriochlorophyll fluorescence at 680 to 690 mμ while the magnesium chelate of 8 hydroxyquinoline was fluorescent at 535 mμ through the Corning No. 110-818 (2A-12). With 3 × slit the interference by the red fluorescence was nullified.

The diluted bacteriochlorophyll sample could theoretically absorb as much as 0.002 optical density units at 770 mμ. To correct the quenching effect of fluorescence, a known amount of magnesium was added to the samples.

Absorption coefficients

Based on the absorbance of the purified bacteriochlorophyll (F-2) and the magnesium contents analyzed, the specific absorption coefficients of the main absorption bands were calculated. Molar absorption coefficients of these bands were calculated taking 911.50 as the molecular weight of bacteriochlorophyll. For the absorption coefficients of pheophytin (F-1), a known amount of bacteriochlorophyll was quantitatively converted to pheophytin by the method described by Smith and Benitez[4] using 25% HCl containing a small amount of ascorbate. The molar absorption coefficients were calculated on the basis of its molecular weight of 889.2.

Table I shows absorption maxima and coefficients of F-1 and F-2 in different organic solvents and

Table II illustrates two main absorption maxima and their ratio of absorbance measured by various authors.

Measurements of fluorescence

The fluorescence of pheophytin and bacteriochlorophyll dissolved in ether were measured using the Baird Atomic Flurospectrometer which was equipped with a near-infrared sensitive RCA 7102 photomultiplier. When the pheophytin was excited at 360 to 380 mμ, the fluorescence maximum appeared at 760 mμ with a shoulder at 693 mμ, but when the same sample was excited at 400 to 420 mμ it gave the fluorescence maximum at 693 mμ with a shoulder at 760 mμ. The excitation spectrum obtained with 693 mμ gave a prominent peak at 420 mμ with a shoulder at 385 mμ besides several minor peaks at 515, 550, and 635 mμ. On the other hand, the excitation spectrum obtained with the 760 mμ emission peak gave a major peak at 363 mμ with minor ones at 535, 635, and 680 mμ. Since the latter excitation spectrum closely resembles the absorption spectrum of the pheophytin, it is evident that the 760 mμ peak is the true fluorescence maximum of the pheophytin.

With thoroughly purified bacteriochlorophyll samples, similar phenomena of fluorescence were encountered. When they were excited at 340 to 380 mμ, a fluorescence peak of 786 mμ with a shoulder of 690 mμ appeared, but when excited at 400 to 440 mμ, the 690 mμ peak became prominent while the 786 mμ peak shrank to its shoulder. The excitation spectrum drawn with the 786 mμ emission band was similar to the absorption spectrum of bacteriochlorophyll so this peak will then be the true fluorescence maximum of bacteriochlorophyll. The latter excitation spectrum gave a 440 mμ maximum and closely resembled the absorption spectrum of the light green photooxidation product, G-2.

The photooxidized light green fractions, G-1, G-2, G-3, and G-4, and the fast migrating gray-green fraction, GG, which were separated on the thin-layer plate, were all highly fluorescent at 686 to 690 mμ area after they were excited at 430 to 446 mμ. A summary of fluorescence data is presented in Table III.

Lipid and fatty acid analysis

The possibility of lipid and fatty acid contamination in the purified samples of bacteriochlorophyll was raised

by Strain and Katz[12] and, therefore, their influences on the absorption coefficients were studied. A gas chromatographic analysis, carried out using a microcolumn with the diethylene glycol adipate as the stainary phase, revealed only traces of oleic and palmitic acids from 136.6 μg of the purified bacteriochlorophyll. In a separate experiment, an absorption study was made using concentrated fatty acids and a triglyceride dissolved in ether, acetone, and methanol. All the absorption values obtained at 770, 675, 577, 391, and 358 mμ with palmitic acid (3.9 mM), stearic acid (0.35 mM), oleic acid (10.6 mM), and triolein (10.3 mM) were within the limits of 0.000 to 0.010.

With much less fatty acid or lipid contaminants possibly mixed in the bacteriochlorophyll samples their influence on the absorption coefficients may well be ignored.

Phase test and HCl number

All the chromatographically pure samples were diluted in diethyl-ether and phase test and determination of HCl numbers were made following procedures described by Smith and Benitez[4]. A summary of absorption coefficients, fluorescence maxima, phase-test data, and HCl numbers is provided in Table IV.

DISCUSSION

The absorption spectra of bacteriochlorophyll and pheophytin, prepared by the described method, agree well with those from preparations by other methods of various workers. A preliminary study of air or photobleaching effects on the infrared (770 mμ) and violet (358 mμ) bands revealed that the former was bleached 1.5 times more rapidly than the latter, even in peroxide-free ether. The maximal band absorption ratio of bacteriochlorophyll (Table II) obtained with the present method was 0.76. The relatively high absorption coefficients of the 770 peak of the bacteriochlorophyll, obtained by the described method, may be related directly to the freshness of the sample. Repeated measurements of the specific absorption coefficient of the infrared peak (778 mμ) in methanol gave only 5.2 $cm^2\ g^{-1}$ (Table I) which is much lower than the previous data, 46.1 $cm^2\ g^{-1}$, published by Smith and Benitez[4].

The appearance of degradation products during the fractionation process on the sucrose column was noted by Smith and Benitez[4], and Holt and Jacobs[3] showed a partial spectrum of a green fraction which resembles the G-2 fraction. Deliberate chemical oxidation as well as photooxidation, followed by complete fractionation and spectral analysis, revealed that bacteriochlorophyll (f-2) was oxidized first to produce a very unstable, but reversible

fraction (F-3) which was further oxidized to bring a green fraction (G-2). Further oxidation of the bacteriochlorophyll produced G-1, G-3, and G-4, but the sequence or mechanism of their production is not yet clear. The catalytic effect of light in oxidation of chlorophyll is apparent since purified chlorophylls in vacuum-sealed glass amples remain unoxidized for years under dim light (Strain and Katz[12]). These green components, however, were similar in absorption and fluorescence spectra, phase test, and HCl numbers. Since the bright green color and the prominent Soret and red bands of the light green components, especially G-2 fraction, closely resemble chlorophyll "a", Fisher and Stern[13] postulated that this could be a dehydrogenated product of bacteriochlorophyll, viz., the 2-acetyl chlorophyll "a". If so, the G-2 and other green fractions may be reduced back to the blue bacteriochlorophyll. Attempts to achieve this reaction with various chemical reductants have been unsuccessful. The absorption spectra of the G-2 and other oxidized components appear quite different from those of bacteriochlorophyll "c" (Jensen, Aasmundrud, and Eimhjellen[9]) "bacterioviridin" (Metzner[14]),

and chlorobium chlorophyll 660 (Larsen[14]). With G-2, absorption maxima were found at 675, 435, and 385 mμ; on the other hand, bacteriochlorophyll "c" showed its absorption peaks at 661 to 662, 431 to 432, and 415 mμ.

The grayish green (GG) fraction was produced in the early stage of air or photooxidation at least as early as the emergence of G-2 and was only accumulating as further oxidation of the bacteriochlorophyll proceeded. No evidence of the conversion of the fraction to other green components was observed. The exact sequential events of these oxidation products of the bacteriochlorophyll are not quite clear at the present time. Kinetic studies to reveal some of the mechanisms involved are in progress.

The corrected fluorescence data obtained from the pheophytin (760 mμ maximum) and bacteriochlorophyll (786 mμ maximum) seem to confirm the earlier data obtained by French[15] who determined that the fluorescence maximum of pheophytin was at 761 mμ and that of bacteriochlorophyll was at 805 mμ with a shoulder at 687 mμ by the excitation at 405 mμ (Smith and Benitez[4]). The appearance of fluoresoence shoulders, 693(s) in pheophytin and 690(s) in bacteriochlorophyll

samples, may certainly be derived from the photooxidized products generated immediately after the irradiation by the strong excitation light and not from the impurity contained in the starting samples. The lability of the bacteriochlorophyll in the light is apparent from the photodegradation experiments in Fig. 2. The close similarity in the patterns of absorption and fluorescence among the light green fractions, G-1, G-2, G-3, and G-4, suggests that there may be only slight differences in the over-all conjugation scheme of the porphyrin nucleus and in some vital side chains. The different fractions may arise by different degrees of allomerization, minor side chain differences or different degrees of solvation of the magnesium ion in the molecule. Further study on the chemical structures of these molecules will be necessary for the satisfactory elucidation of the physical phenomena exhibited by these fractions.

The positive phase-test results with various fractions of bacteriochlorophyll (Table IV) may well be disputed, since it is unlikely that the oxidized G series as well as the GG fraction could remain totally unallomerized. The positive

phase test may not necessarily mean free of allomerization (Smith and Benitez[4]), and it may fail only when considerable fraction of the chlorophyll has been allomerized.

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TABLE I

ABSORPTION MAXIMA AND COEFFICIENTS OF BACTERIOCHLOROPHYLL AND PHEOPHYTIN IN VARIOUS SOLVENTS

Maximum wavelength	Bacteriochlorophyll in Ether		Acetone		Methanol		Pheophytin in Ether	
(mμ)	*10^{-3}e	**k	10^{-3}e	k	10^{-3}e	k	10^{-3}e	k
778					4.7	5.2		
775			18.8	22.1				
770	98.7	108.3						
750							73.3	80.4
680							16.8	18.4
676			17.2	18.9				
616							5.0	5.5
587					21.2	23.3		
575	22.2	24.4	9.3	10.2				
528							27.9	30.7
393					52.0	57.1		
391	57.6	63.2						
387							61.2	67.1
358	75.1	82.5	40.3	44.2				
357							120.8	132.6
333					49.8	54.7		

*10^{-3}e: Millimolar absorption coefficient, $cm^2 \times mmole^{-1}$

**k: Specific absorption coefficient, $cm^2 \times g^{-1}$

TABLE II

ABSORPTION COEFFICIENTS OF TWO MAXIMA OF BACTERIOCHLOROPHYLL AND THEIR RATIO MEASURED IN ETHER

Author	$*\lambda_I$ (mμ)	$10^{-3}e$	k	$*\lambda_V$ (mμ)	$10^{-3}e$	k	λ_V/λ_I
Weigl[16]	772	95.6	105	358	85.3	93	0.89
Holt and Jacobs[3]	769 770	93.4	102	357	80.8	77	0.75
Smith and Benitez[4]	773	91.1	100	358.5	73.3	80	0.80
Kim and Feller[10]	770	98.7	108	358	75.1	82	0.76

$*\lambda_I$, λ_V: Maximum wavelengths of infrared and violet bands, respectively.

TABLE III

EXCITATION AND FLUORESCENCE MAXIMA OF BACTERIOCHLOROPHYLL AND ITS DEGRADATION PRODUCTS IN ETHER

Sample	Excitation (mμ)	Fluorescence (mμ)	
Pheophytin	363	693(s)*	760
	420	693	760(s)
Bacteriochlorophyll	370	690(s)	786
	440	690	786(s)
Light green fractions:			
G-1	436	688	
G-2	440	690	
G-3	435	686	
G-4	440	690	
Gray-green fraction:			
GG	436	686	

*(s): Shoulder

TABLE IV

SUMMARY OF PHYSICAL DATA OBTAINED FROM FRACTIONS OF BACTERIOCHLOROPHYLL

Fraction	Principal absorption maxima in ether		Fluorescence maxima in ether	Phase test	HCl. number
	Mμ	10^{-3}e			
Pheophytin	750	73.3	760	+	32
(F-1)	528	27.9			
	357	120.8			
Bacterio-	770	98.7	786	+	30
chlorophyll	575	22.2			
	358	75.1			
Light green	675	40.8	690	+	30
fraction (G-2)	435	50.4			
Gray-green	675	11.3	686	+	30
fraction (GG)	415	32.7			
	330	46.0			
	280	86.1			

FIGURE LEGENDS

Figure 1.- Fractionation of bacteriochlorophyll.

Figure 2.- Thin-layer chromatography of bacteriochlorophyll and its degradation products. (c) Carotenoids; (F-1) pheophytin; (F-2) bacteriochlorophyll; (F-3) gray-blue fraction; (GG) gray-green fraction; (G-1), (G-2), (G-3), (G-4) green fractions.

Figure 3.- Absorption spectra of bacteriochlorophyll (F-2) and its degradation components, (F-1) pheophytin; (G-2) oxidized green component; (GG) gray-green component.

Figure 4.- Absorption spectra of bacteriochlorophyll (F-2) and the gray-blue fraction (F-3).

Sample (1.0-ml packed or 100-mg dried cells)

↓ Suspend in 20-ml MeOH containing 1-ml 1M ascorbate centrifuge immediately at 30 000 g × min. → Precipitate

Supernatant

↓ Take up in 250-ml petroleum ether, reduce volume to 2.0 ml

Diatomaceous Earth Column (1.0 × 20 cm)

↓ Dry pack Gas Chrom P (120-140 mesh), place sample on top, wash with cold 1% acetone in pet ether (100 ml, v/v) discard

↓ Wash with 3% acetone/pet ether (v/v) (50 ml) discard

↓ Elute with 5% acetone/pet ether (v/v) (100 ml) (F-1: violet)

↓ Wash with 5% acetone/pet ether (v/v) (50 ml) discard

↓ Elute with 7% acetone/pet ether (v/v) (100 ml) (F-2: blue)

↓ Elute with 10% acetone/pet ether (v/v) (100 ml) (F-3: gray-blue)

Vacuum dry F-1, F-2, F-3, and dissolve in pure solvents

Fig 1

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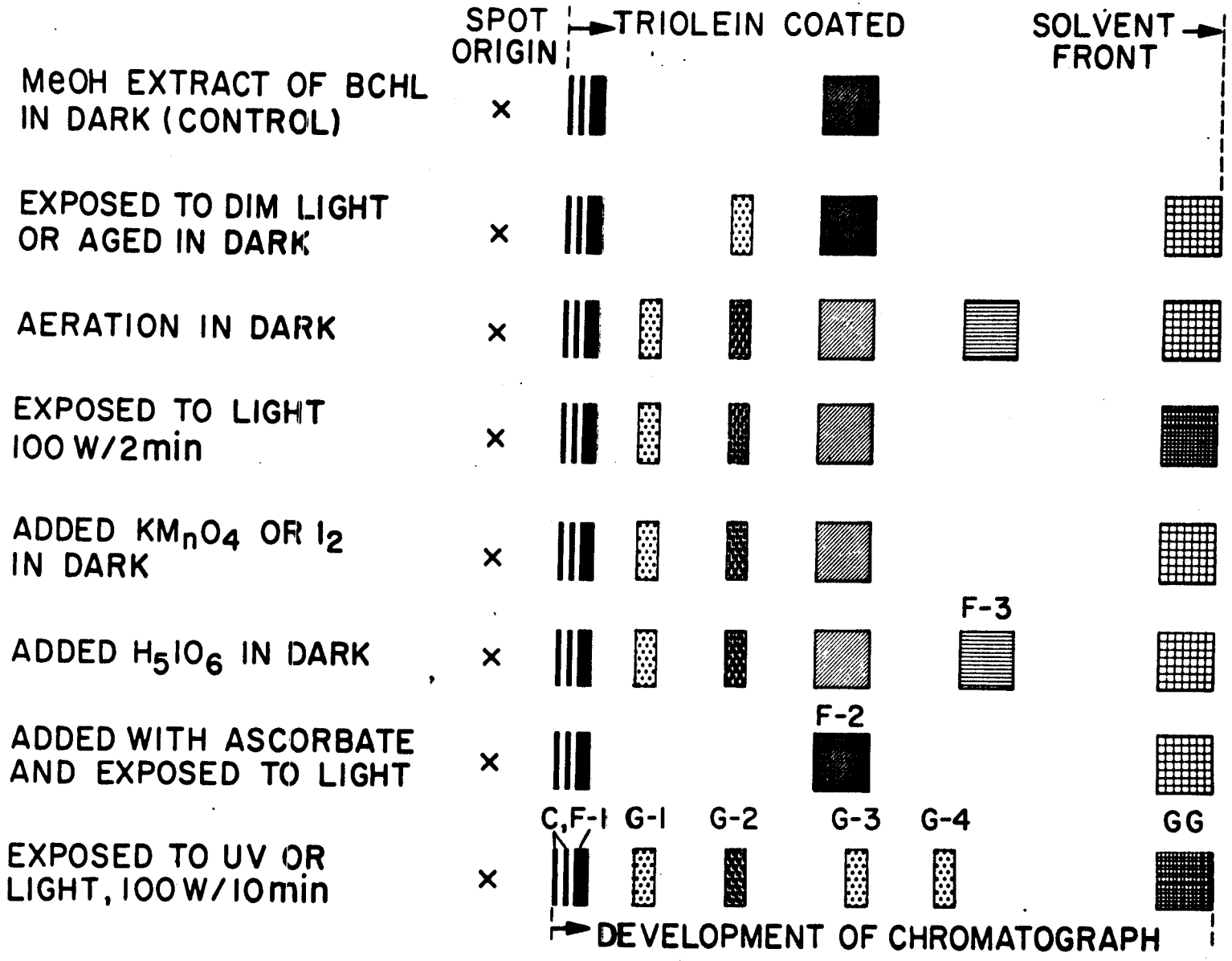

Fig 3

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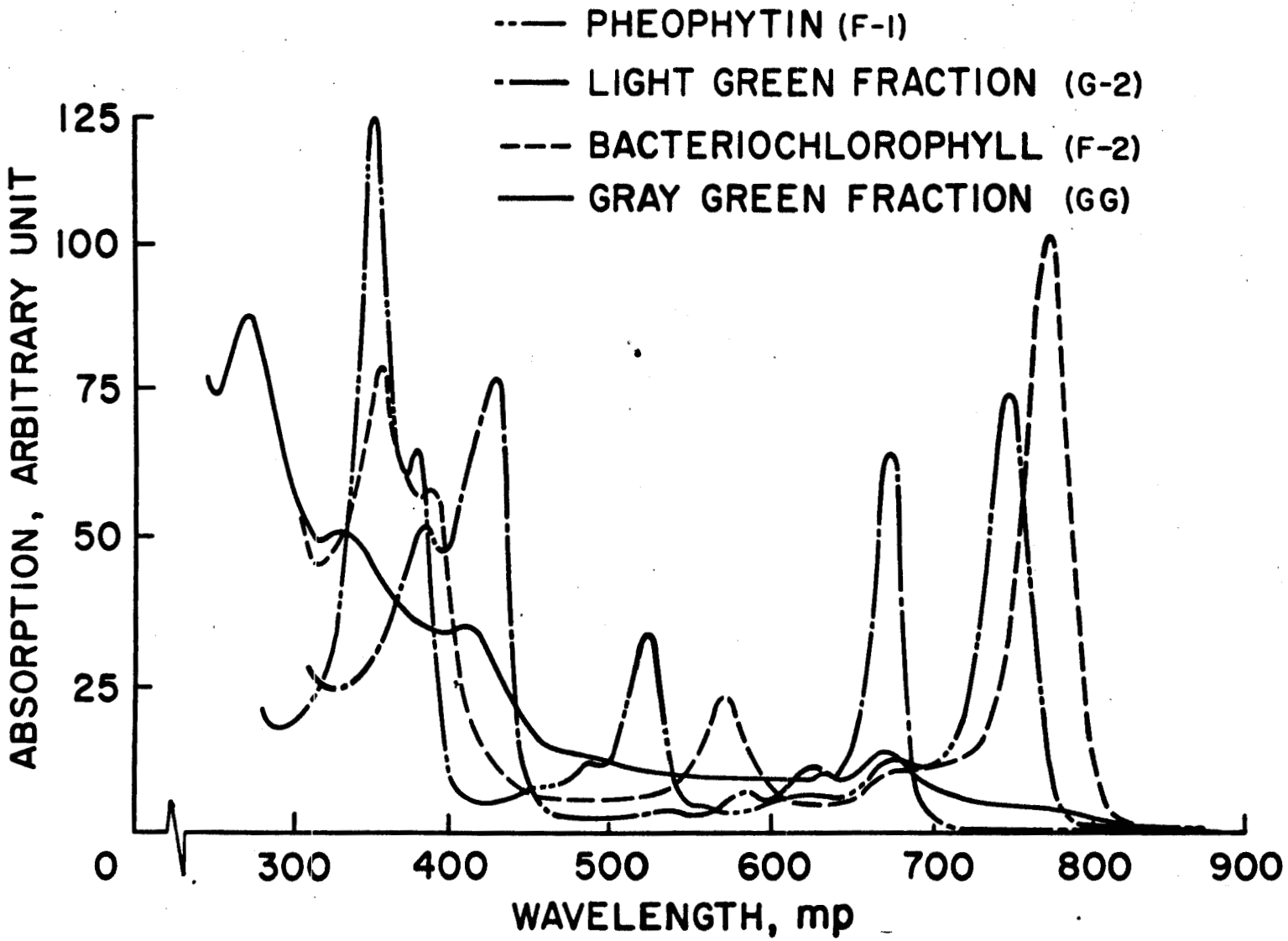

Fig 3

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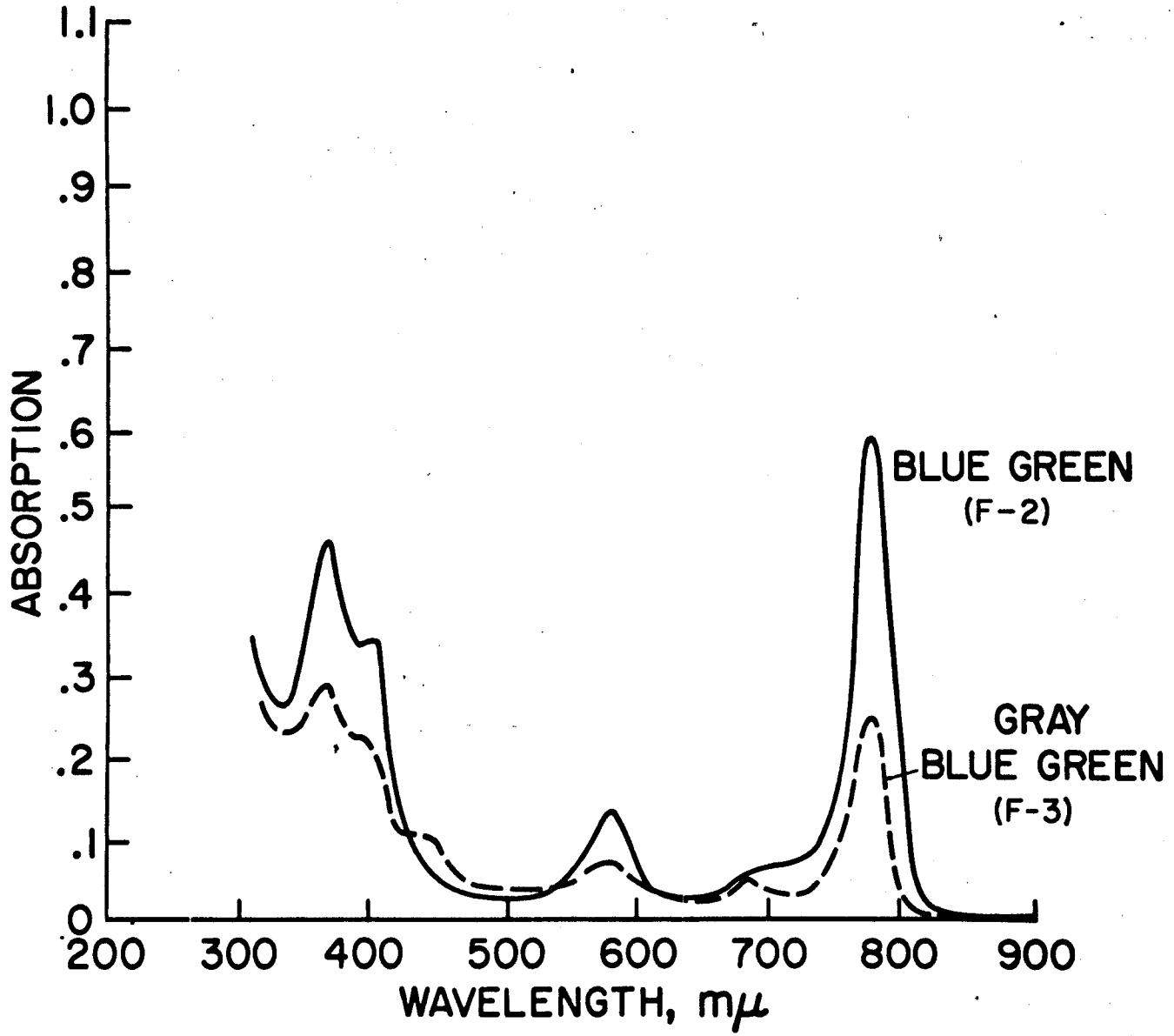

Fig 4

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